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Chronically Implanted, Nafion-Coated Ag/AgCl Reference Electrodes for Neurochemical Applications

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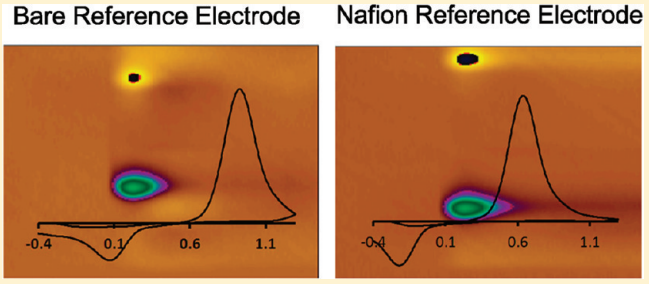
ABSTRACT: Fast-scan cyclic voltammetry at carbon-fiber microelectrodes can be used to measure behaviorally correlated dopamine changes in the extracellular fluid of the brain of freely moving rats. These experiments employ a chronically implanted Ag/AgCl reference electrode. When dopamine measurements are taken 4 days after implantation, there is often a potential shift, typically greater than +0.2 V, in the anodic and cathodic peaks in the cyclic voltammogram for dopamine. In this work, we optimized a method to coat sintered Ag/AgCl reference electrodes with the perfluorinated polymer, Nafion, to prevent this shift. We find that we can stabilize reference electrodes for up to 28 days. Immunohistochemistry of the tissue around the implant site shows extensive glial encapsulation around both bare and Nafion-coated devices. However, the lesion around bare electrodes has a rough texture implying that these cells are strongly adsorbed onto the bare reference electrode, while the lesion around a Nafion-coated electrode shows that cells are more intact implying that they adsorb less strongly. Energy dispersive X-ray spectroscopy and scanning electron microscopy analysis of the surface of the electrodes confirms this by visualizing a heavy buildup of plaques, organic in nature, only on bare electrodes. Impedance spectroscopy indicates no difference between the impedance of bare and Nafion-coated Ag/AgCl electrodes, indicating that glial encapsulation does not lead to an increase in uncompensated resistance between the working and reference electrodes. The electrochemical shift therefore must be due to the unique chemical microenvironment around the reference electrode that alters the chloride equilibrium, a process that the Nafion coating prevents.



KEYWORDS: Astrocytes, glia, fast-scan cyclic voltammetry, carbon-fiber microelectrode, dopamine, in vivo, neuroinflammation, peak shift, surface analysis, impedance spectroscopy

Fast-scan cyclic voltammetry (FSCV) is a powerful tool for studying in vivo, subsecond neurotransmitter dynamics.[1−4] Miniaturization of the electronics associated with FSCV has allowed correlation of transient dopamine concentration fluctuations with behavior in freely moving rats.[5,6] These behavioral experiments necessitate a "survival" surgery in which an electrical stimulating electrode, a guide cannula, and a Ag/AgCl reference electrode are permanently implanted into the brain of a rat. After a recovery period (typically 4 days), the rat is placed in an operant chamber and a fresh carbon-fiber electrode is lowered into the brain for voltammetric recordings of dopamine during behavior. These recordings often yield cyclic voltammograms where the oxidation and reduction potentials associated with dopamine redox processes are "shifted" with respect to in vitro recordings. This electrochemical shift, typically of +0.2−0.3 V, can have dramatic effects on the voltammetric measurements. First, the identification of the species electrolyzed, which is based upon the position of the voltammetric peaks, is confounded. Second, the sensitivity of the responses is altered because the voltammetric response to adsorbed species depends upon the potential limit of the voltage scan.[7] These electrochemical shifts arise from a drift in response of the chronically implanted reference electrode because they are not seen with acute implantation.[8,9]

Deterioration of probes permanently implanted in the brain is commonly seen in a variety of experiments,[10−12] and the brain's response toward implanted devices has been well-documented.[13−16] When a foreign body is inserted into the tissue, a dense network of glial cells encapsulates the device, isolating it from the tissue in a process called gliosis or glial scarring.[17−19] For FSCV measurements, the electrochemical shift affects the quantification of dopamine levels and reproducibility of substrate identity. In the past, we have offset the potential shift. While this approach is effective, it is desirable to prevent the shift from occurring so that the potentials employed in the brain are accurate.

In this paper, we demonstrate a method that minimizes this electrochemical shift. Past research has focused on strategically shaping, texturing, coating, or optimizing materials for devices to reduce tissue responses.[10,20−23] In 1994, Moussy and Harrison found that the rapid subcutaneous degradation of Ag/AgCl could

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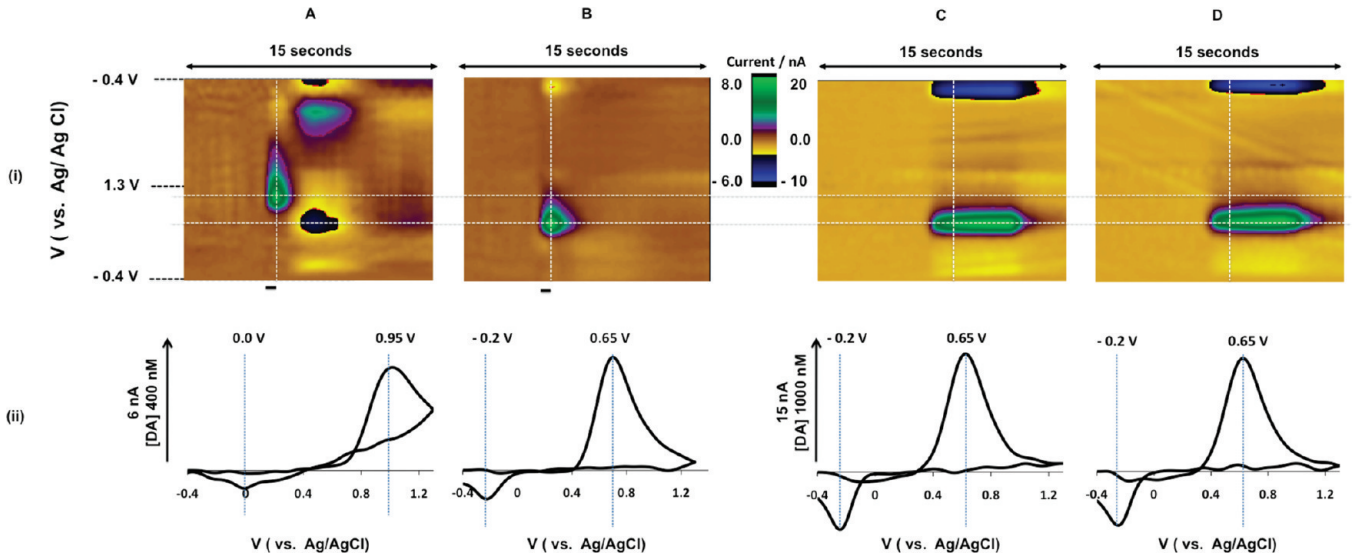

Figure 1. (i) Color plots with potential on the *y*-axis plotted against time on the *x*-axis and the current response represented in false color. These plots represent (A) the signal obtained in the NAc of a freely moving rat upon MFB stimulation (black bar under the color plot denotes the stimulation time and duration) 4 days after initial surgery, (B) the same signal after a −0.3 V "offset", (C) an in vitro injection of dopamine (1 μM) vs a fresh Ag/AgCl reference electrode, and (D) an in vitro injection of dopamine (1 μM) vs the Ag/AgCl electrode explanted from the rat used in signal (A). (ii) (A−D) Representative cyclic voltammograms taken from the vertical white dashed lines.

be prevented with Nafion modifications to the reference electrode surface.[24] Here, we optimize their method and apply it to in vivo neurochemical FSCV. We found that we could stabilize our reference electrodes with this method for up to 28 days. Via immunohistochemistry of the tissue at the implant site and surface analysis of the electrodes, we found that the mechanism of this increased stability was consistent with the extent of glial cells physically adhered onto the electrode's surface, a process that Nafion prevents. Using electrical impedance measurements, we found that this effect is not electrical but rather most likely due to chemical changes in the microenvironment of the glial cells.

RESULTS AND DISCUSSION

Electrochemical Shift in Peaks for Dopamine Oxidation and Reduction 4 Days after Implantation. FSCV experiments in behaving rats require a period of recovery after the surgery to implant the reference and stimulating electrodes. Within this 4 day period, chemical or physical processes occur around the reference electrode surface such that when dopamine is measured with a fresh carbon-fiber microelectrode, the oxidation and reduction potentials are "shifted" with respect to their in vitro positions. Figure 1A illustrates this type of response in a freely moving rat that had undergone surgery 4 days previously. The color plot in panel (i) displays cyclic voltammograms collected at 10 Hz for 15 s in the NAc during an electrical stimulation (at 5 s) of the MFB. The time of the electrical stimulation is indicated with the black bar under the color plot. This electrically evoked response has previously been characterized anatomically, physiologically, chemically, and pharmacologically to arise from neuronal release of dopamine.[1] However, the peaks for this substance recorded in vivo do not occur at the same potential as those for authentic dopamine (1 μM) measured in vitro (Figure 1C) using a freshly fabricated Ag/AgCl electrode. This is also apparent in the corresponding cyclic voltammograms shown in panel (ii) of Figure 1. In vivo, the oxidation occurs at around 0.95 V instead of 0.65 V and the reduction occurs at 0.0 V instead of −0.2 V, showing that a process at the implanted reference electrode has offset the potential of the applied waveform. Moreover, this change in waveform seems to enhance sensitivity to the pH changes that occur after the stimulation, making it more difficult to distinguish smaller dopamine signals. We have previously compensated for this by applying a counter potential offset as illustrated in Figure 1B where the horizontal white dashed lines show the position of the maximum oxidation with respect to the in vitro signal. In this example, the waveform was offset by −300 mV, which shifts the oxidation response to 0.65 V and the reduction to −0.2 V. This response then matches the in vitro response (Figure 1C).

Interestingly, the potential of this "shifted" reference electrode is not permanently altered. To establish this, we removed the reference electrode from the rat's brain and used it as a reference electrode in vitro for dopamine measurements. Figure 1D shows that, despite the shifted electrochemical response in vivo, the reference electrode yields an electrochemically characteristic dopamine cyclic voltammogram that is not offset in vitro. Thus, the mechanisms leading to the electrochemical shifts are specific to the local environment around the reference electrode in vivo.

Prevention of Electrochemical Shift with Nafion-Coated Ag/AgCl Reference Electrodes. In our previous work, and in the example shown in Figure 1, we constructed Ag/AgCl reference electrodes by electroplating AgCl onto a silver wire.[25] Because this resulted in a thin chloride layer, it may be particularly susceptible to an alteration in chemical composition when implanted in the brain. Thus, we used commercially available, sintered Ag/AgCl electrodes (E255A, In Vivo Metric, Healdsburg, CA). The Ag/AgCl is sintered in a mesh matrix, and a Ag wire is forced into the matrix to create the electrode that has a much thicker AgCl coating than the homemade reference electrodes. However, despite the thicker chloride layer, these electrodes also showed a potential shift of similar magnitude in vivo 4 days after implantation.

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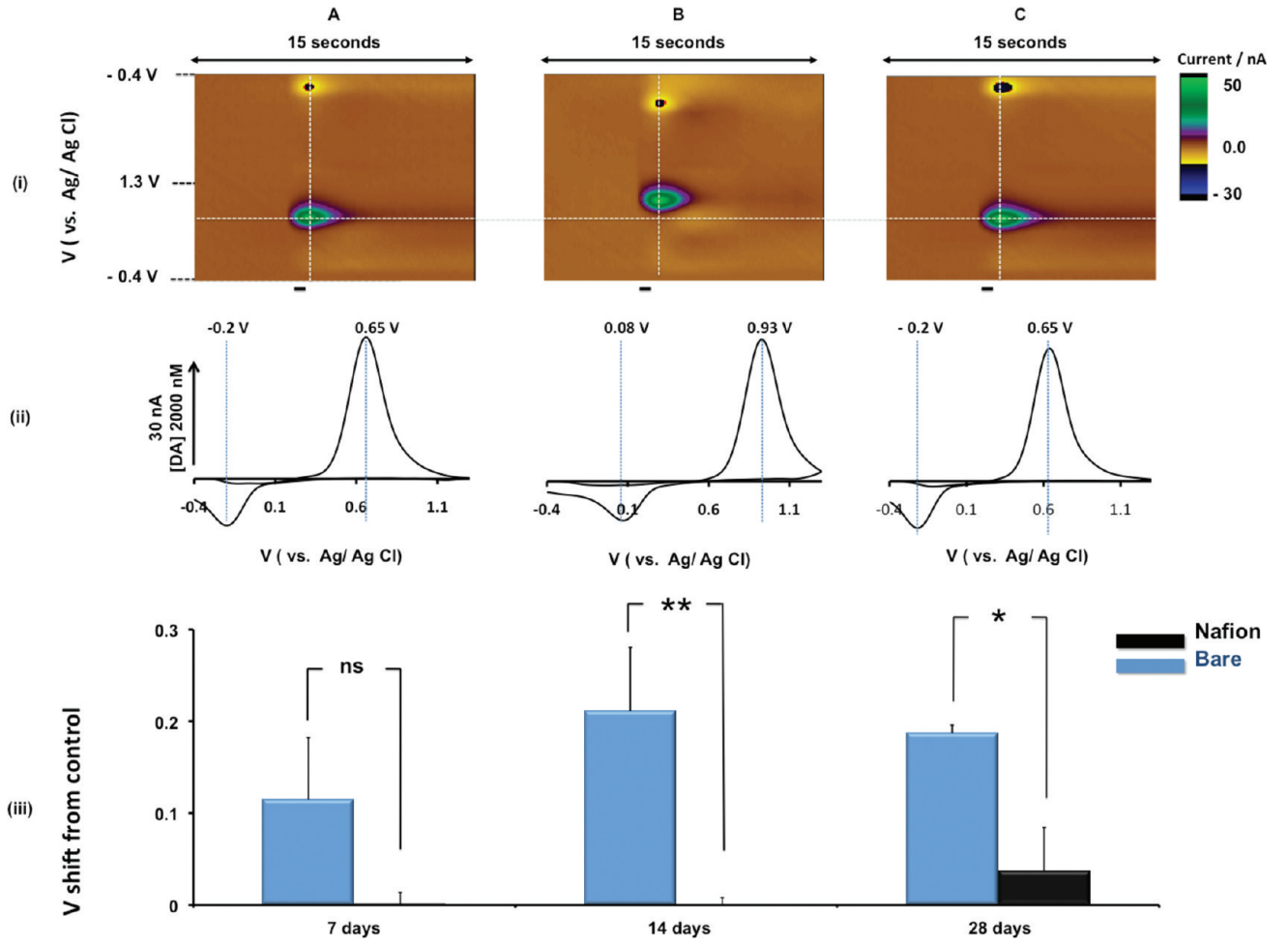

Figure 2. (i) Color plots with potential on the *y*-axis plotted against time on the *x*-axis and the current response represented in false color. These plots represent (A) a dopamine signal in the NAc of an anesthetized rat upon MFB stimulation (stimulation time and duration noted by black bar under the plot) vs an acutely implanted Ag/AgCl reference electrode, (B) a dopamine signal in the same rat vs a bare electrode implanted for 7 days, (C) a dopamine signal in the same vs a Nafion-coated electrode implanted for 7 days. (ii) (A−C) Representative cyclic voltammograms taken from the vertical white dashed lines. (iii) Averaged volts shifted from an acutely implanted Ag/AgCl reference electrode for bare (blue) and Nafion-coated (black) electrodes for 7, 14, and 28 days ($n = 6$ per group (18 in total) ± SEM). Results were analyzed using a two-way ANOVA showing a significant difference in potential shift between bare and Nafion electrodes ($P < 0.001$) and post-tests to compare groups at the different time points; $^{*}P < 0.05$, $^{**}P < 0.01$.

Moussy and Harrison previously described Nafion coatings on Ag/AgCl reference electrodes that prevented the degradation of their electrochemical performance.[24] We have previously utilized Nafion extensively in our electrochemical recordings;[3,26,27] therefore, we optimized their coating technique in an attempt to stabilize the potential of our reference electrodes. The sintered electrodes were used because their rough surface is ideal for adherence of polymer coatings. The electrodes were dipped five times in 5% Nafion solution for 10 s with a slow agitating motion, and each coating was allowed to dry for 30 min. These electrodes were dried in air overnight and then cured at 120 °C for 1 h. To test their stability, we performed experiments where we implanted both Nafion and uncoated reference electrodes into the brains of rats who were then allowed to recover for 7, 14, and 28 days. After these periods, the rats were anesthetized, an acute reference electrode and carbon fiber electrode were implanted into their brain, and dopamine release was compared with recordings using each of the three reference electrodes (acute, Nafion-coated, permanently implanted and bare, permanently implanted). Figure 2A (i) shows a color plot of the dopamine response in the NAc of an anesthetized animal upon MFB stimulation when the potential was applied versus the acutely implanted Ag/AgCl reference electrode. Figure 2B (i) and C (i) shows comparisons of this same response, at the same carbon-fiber microelectrode in the same rat, however, versus bare (B) and a Nafion-coated (C) chronically implanted reference electrodes that had been implanted for 7 days. Panel (ii) shows the corresponding cyclic voltammograms. As before, the bare electrode displays shifted oxidation and reduction peaks (0.93 and 0.08 V, respectively); however, the Nafion-coated reference electrode prevents this effect. The positions of the oxidation and reduction peaks are maintained and correlate not only with the acutely implanted reference electrode, as indicated by the horizontal white dashed line, but also to an in vitro experiment (0.65 and −0.2 V, respectively). This effect is maintained over multiple days. Figure 2 (iii) shows a histogram that compares dopamine potential shifts between acutely implanted reference electrodes and bare (blue) and Nafion-coated (black) electrodes that have been implanted into the brain for 7, 14, and 28 days ($n = 6$ for each group (18 in total) ± SEM). The bare electrodes display shifts in all cases,

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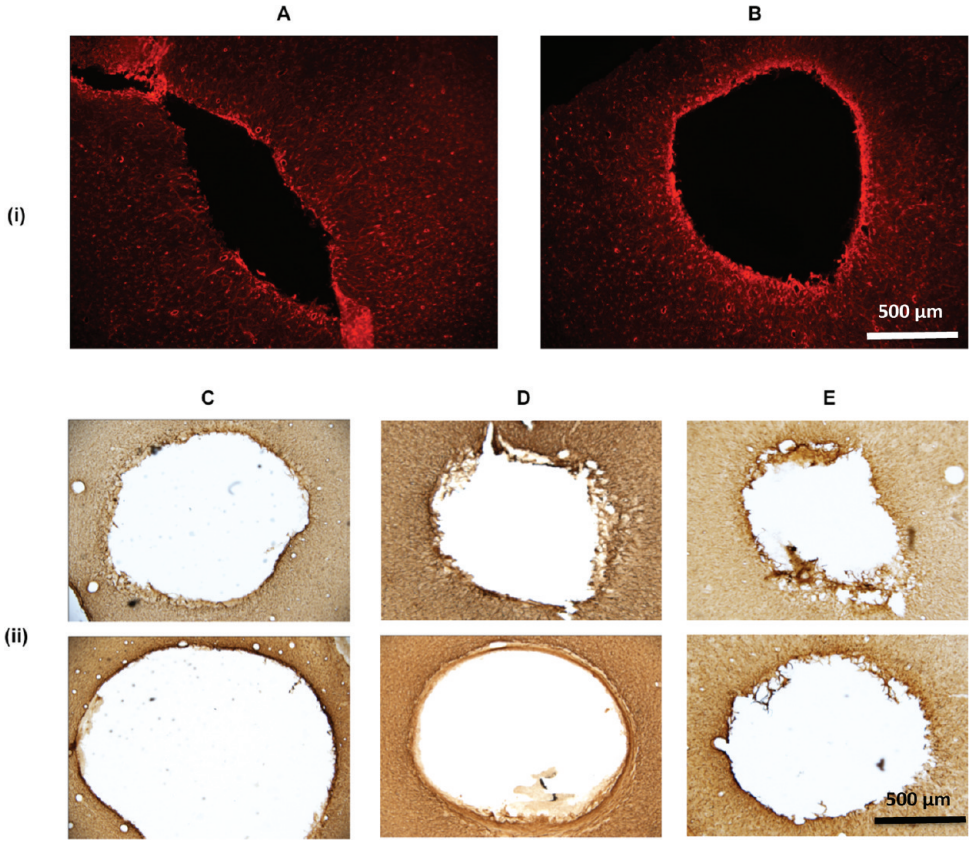

Figure 3. Glial fibrallary acid protein (GFAP) immunoreactivity in cortical astroglia after reference electrode removal. (i) Fluorescence images of the tissue around the implant site of (A) a bare Ag/AgCl reference electrode and (B) a Nafion-coated electrode after 14 days. (ii) Bright images of GFAP immunoreactivity around representative lesion sites for bare (top) and Nafion-coated (bottom) electrodes for 7 days (C), 14 days (D), and 28 days (E).

most robustly after 28 days as evidenced by the small error margin. The Nafion coated electrode reduces this shift, however. We analyzed the shifts in peak potential using a two-way ANOVA, with appropriate post-tests, and found that Nafion coatings had a significantly different behavior than the bare electrodes on potential over time ($P < 0.001$). Using the post-tests, it was determined that there was no significant difference between the groups after 1 week implantation ($P > 0.05$), but a significant difference after 2 and 4 weeks of implantation ($P < 0.01$ and $P < 0.05$, respectively). We can therefore increase the stability of the reference electrodes robustly and significantly for up to 28 days using this technique.

Immunohistochemical Analysis of Implant Site in Tissue. To better understand how Nafion coating prevents the electrochemical shift, we performed immunohistochemical analysis of the cortical tissue around the reference electrode implantation site. Figure 3 (i) shows fluorescence images of antibody binding to glial fibrillary acid protein (GFAP) around the lesion site where the reference electrodes were explanted after 14 days for (A) bare and (B) Nafion-coated electrodes. GFAP is expressed only in glial cells; therefore, a higher degree of fluorescence corresponds to a larger density of glial cells. Figure 3 (ii) shows extensive immunoreactivity to GFAP antibody. Glial scarring is present in the lesion surrounding the implantation site of both Nafion-coated and bare reference electrodes. However, the lesion sites of Nafion-coated electrodes (B) have a qualitatively different appearance around the implant border after the electrode is removed. The implant border of the lesion around the bare electrodes is rough in appearance and implies that the cells are disrupted or torn after electrode removal. The implant border of the lesion around the Nafion-coated electrodes has a smooth appearance, implying that cells are less disturbed by electrode removal. This difference is also apparent in the bright field images in Figure 3 (ii) that compare bare (top) versus Nafion-coated (bottom) electrodes after 7 days (C), 14 days (D), and 28 days (E) post-implantation. In all cases, the implant borders of the lesions left after removal of the Nafion-coated electrodes have a smoother appearance, showing more cells intact. The difference in roughness around the lesion sites may be due to the Nafion's hydrophobic properties that prevent glial cells from adhering to the electrode surface. The extent of glial scarring is dependent on the dimensions of the device,[28] and since the bare and Nafion-coated electrodes are equivalent in size, the immune response to them should be comparable. However, because the cells adhere strongly to the bare-electrode surface, once this electrode is removed, they may effectively be "ripped" out during electrode removal. The glial cells however adsorb less strongly to the Nafion surface; hence, when this electrode is removed, more cells remain intact in the tissue. This appears to be the case up until 28 days where the implant border of the lesion left after removal of the Nafion-coated electrode (E) also appears rough. This agrees with the voltammetric data that showed electrochemical shifts beginning to occur at 28 days. Therefore, the extent of intact glial cells around the lesion site of the electrode is consistent with the electrochemical shift.

SEM/EDS Analysis of Electrode Surface. We next analyzed the surface of the reference electrodes after their removal from

the brain with scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). Figure 4 contains SEM images of bare (i) and Nafion-coated (ii) electrodes before implantation (A) and after 28 days (B) in the rat brain. Before implantation, there is a clear visual difference between the bare electrode and the Nafion-coated electrodes. The bare electrodes have a coarse appearance due to the sintered Ag/AgCl surface, while the Nafion-coated electrodes appear smoother. The shiny appearance of the Nafion is easily distinguishable from the Ag/AgCl. As implantation time is increased, a buildup of large plaques is apparent on the bare electrode surface; this is particularly striking after 28 days (B) and is highlighted with the focused image in (C). In contrast, the Nafion-coated surfaces (ii) remain free of plaques. This is consistent with the histological data that shows that the cells remain intact in the tissue and indeed with the voltammetric data that shows no electrochemical shifts. However, as implantation time is increased up to 28 days (B), the Nafion-coated electrode resembles a bare electrode, showing that the Nafion coating has degraded. The 28-day marker is again consistent with the voltammetric data that shows a small percentage of electrodes that shift after 28 days and with the histological data that shows a rough surface lesion around the Nafion-coated electrode's implant site in tissue.

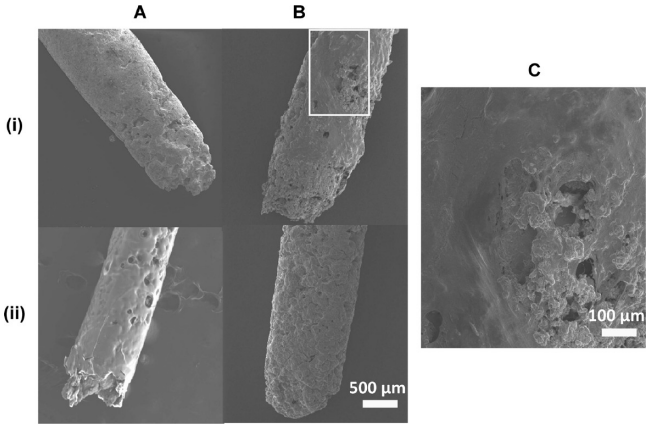

Figure 4. Representative scanning electron microscopy images of bare (i) and Nafion-coated (ii) electrodes before implantation in the brain (A) and after 28 days implantation (B) in the brain, and (C) zoomed section as outlined by the white box.

We next chemically analyzed three distinct sites on the electrode's surface with EDS. EDS displays high spatial resolution with an ability to measure the elemental content of the material at depth of several micrometers. Hence, we can observe the Ag present under the Nafion coatings. Figure 5 shows representative EDS spectra for (A) a bare electrode pre-implantation, (B) a Nafion-coated electrode pre-implantation, (C) a bare electrode after 14 days post-implantation, and (D) a Nafion-coated electrode 14 days post-implantation. Peaks were quantified as the atomic percent of each species observed. The Nafion-coated electrodes display the same peak positions as the bare electrodes (Ag doublet at 3 keV; Cl peaks at 0.1 and 2.8 keV) and in addition have peaks for carbon (peak at 0.3 keV), sulfur (peak at 2.3 keV), and fluorine (peak at 0.7 keV), key components of the nonpolar perfluorinated coating. After 14 days, additional peaks can be observed on the bare electrodes including carbon and oxygen (peak at 0.6 keV). This confirms that the composition of the hard plaques apparent in Figure 4 are organic, providing yet more evidence that cells are adsorbing to the bare electrode surface. We can quantify the Nafion levels on the electrode surface by measuring the atomic percent of fluorine as a function of implantation time. Figure 5E shows that the content of fluorine on the surface of the electrode is around 60% before implantation. After 7 days, the content is around 50% ($P < 0.05$ compared to pre-implantation). At 14 days, the fluorine content is around 30% ($P < 0.001$ compared to pre-implantation) and dramatically lower after 28 days at around 7% ($P < 0.001$

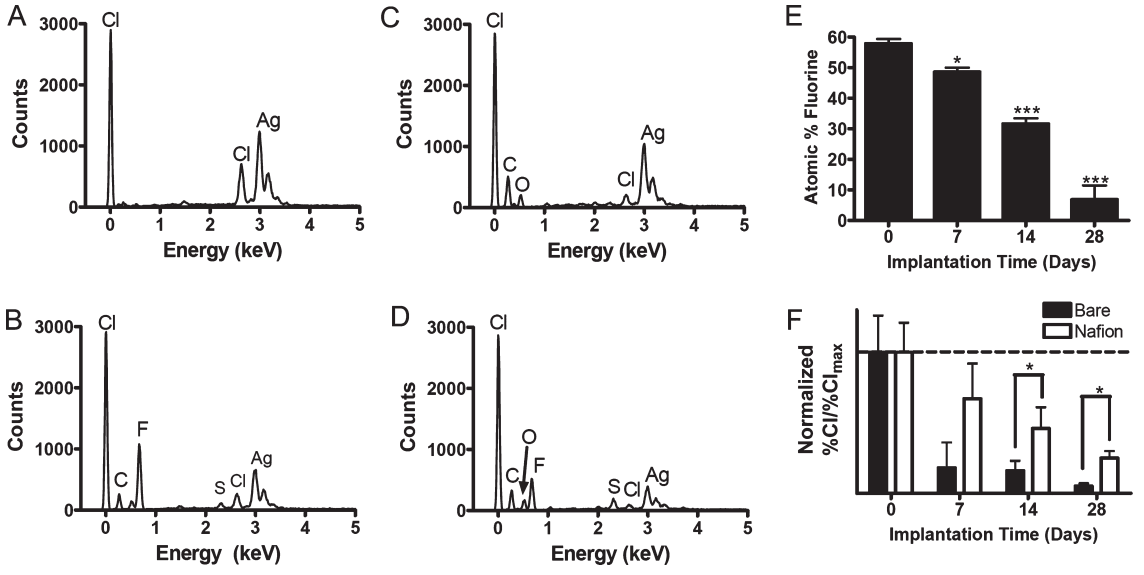

Figure 5. Representative EDS spectra for Ag/AgCl reference electrodes uncoated before brain implantation (A), Nafion-coated before brain implantation (B), uncoated after 2 weeks brain implantation (C), and Nafion-coated after 2 weeks brain implantation (D). Peaks are labeled indicating the atom they represent. (E) Atomic percent fluorine on the surface of Nafion-coated electrodes after 0, 7, 14, and 28 days brain implantation (*$P < 0.05$, ***$P < 0.001$, one-way ANOVA with appropriate post hoc test compared to 0 days). (F) Normalized, atomic percent chlorine on the surface of Nafion-coated (white) and bare (black) electrodes after 0, 7, 14, and 28 days brain implantation (Student's t test was performed on unpaired data; *$P < 0.05$ was taken as significant).

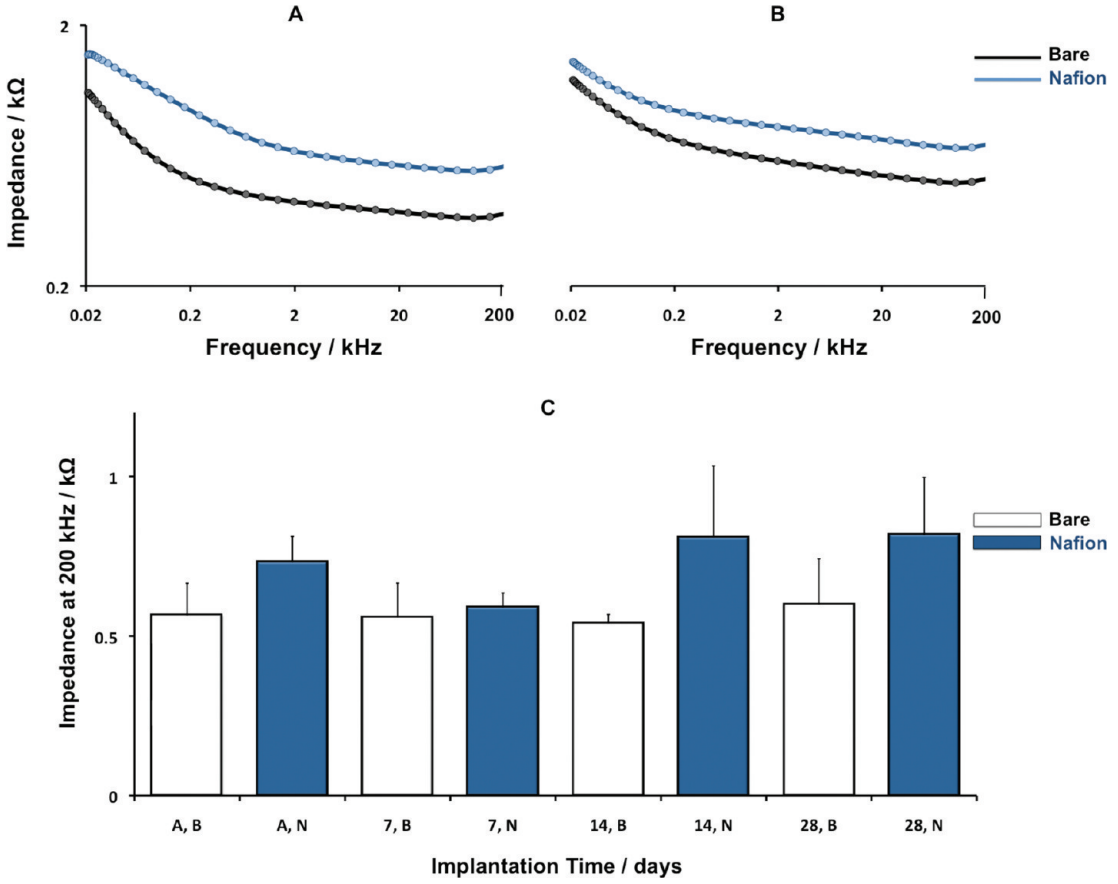

Figure 6. (A) Impedance of reference electrodes: Frequency dependent impedance of the reference electrodes measured versus acutely implanted Ag/AgCl electrodes right after implantation (0 days) and after 28 days of chronic implantation (B) for bare and Nafion-coated electrodes. (C) Time course of changes in impedance at 200 kHz for bare (B) and Nafion-coated (N) electrodes immediately after implantation (0, B and 0, N), after 7 days (7, B and 7, N), after 14 days (14, B and 14, N), and after 28 days (28, B and 28, N), respectively ($n = 3$ rats for each data point). Data were analyzed with a two-way ANOVA with post-tests to compare groups; $P > 0.05$ for all comparisons showing no significant differences in impedance between groups over various implantation times.

compared to pre-implantation). These data quantitatively support the visual observation from Figure 4 that the Nafion coating can fail after 28 days. Furthermore, when we do the same analysis for chlorine on bare versus Nafion-coated electrodes (Figure 5F), we find a decrease in the chlorine levels in both cases. When normalized, the chlorine levels are relatively more abundant after 14 and 28 days post-implantation on the Nafion coated electrodes as compared to the bare electrodes. This implies that the electrochemical shift is dependent on a critical level of chloride on the surface. Because Nafion is a cation-exchange polymer, it is likely that it protects the chloride ion equilibrium at the reference electrode surface. Accordingly, EDS analysis of the Nafion-coated surface shows that although the absolute levels of chlorine are smaller on the Nafion-coated surface (due to the Nafion coating), the levels drop less significantly at 14 and 28 days ($p < 0.05$).

The data from these studies support the notion that the Nafion coatings on reference electrodes are stable up to 28 days in the rat brain. Cell adsorption onto the reference electrodes is consistent with the loss of chlorine on the surface and subsequently with the electrochemical shift. Viable Nafion coatings prevent cell adsorption, maintaining the chlorine layer, hence preventing electrochemical shifts.

Origin of Electrochemical Peak Shift. A peak shift in cyclic voltammograms can have several origins. The recordings shown here were done with freshly implanted carbon fiber electrodes employing a uniform scan rate[29] and constant filtering, so these factors and changes in electron transfer kinetics are not responsible for these observations. Changes in the media resistance or a change in the standard electrode reference potential are two other possible causes. The formation of the glial scar and inevitable encapsulation of the foreign body by astrocytes at chronically implanted electrodes[17−19] both could increase the impedance.[30] Therefore, we measured the impedance of bare (black) and Nafion-coated electrodes (blue) for acutely implanted electrodes in Figure 6A and after 14 days implantation in Figure 6B. We found negligible differences in the impedance between these two electrodes at all frequencies. A two-way ANOVA with post-tests showed no significant difference between the impedance of bare and Nafion coated electrodes over 7, 14, and 28 days ($n = 3 \pm$ SEM each group, $P > 0.05$ for all data). Furthermore, Figure 6C shows the impedance value at 200 kHz where the impedance is dominated by the resistance of the media. Collectively, these data show that the increased gliosis around the reference electrode does not lead to increased resistance between the working and reference electrodes.

Thus, the most likely origin of the potential shift with chronically implanted bare reference electrodes is a chemical change in or around its surface. Our EDS data (Figure 5F) reveal

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a decrease in available surface chlorine of the bare electrodes in the brain, consistent with this hypothesis. However, reference electrodes that yield shifted cyclic voltammograms in vivo do not demonstrate the shift once explanted and used in vitro (Figure 2). This may be because the adsorbed cells wash off the explanted electrode in vitro, exposing more of the Ag/AgCl. Alternatively, the artificial cerebrospinal fluid used as the buffer in vitro may replenish the choride layer. These factors would reinstate the chloride equilibrium of the Ag/AgCl.

These data reveal that there is a change in the functional chemical microenvironment around the reference electrodes in vivo. Based on the literature documenting the roles of glial cells in immune-defense, there are two possible mechanisms. First, the formation of a glial scar is thought to create an ion-impermeable barrier, much like the blood-brain barrier around the implanted device.[31] This barrier is largely impermeable to ions and may effectively shift the ionic equilibrium near the reference electrode surface. Nafion, a cation exchange polymer, could create an anionic (Cl^-) buffer zone between the glial scar and the electrode surface. Alternatively, glial cells do not passively encapsulate the device, but rather they actively secrete cytotoxic chemicals onto the device in order to inactivate it. These include nitric oxide,[32] reactive oxygen intermediates (ROIs), hydrogen peroxide, and superoxide.[33] These chemicals could disrupt the Cl^- equilibrium of the reference electrode; Nafion's cation exchange properties would repel some of these species, in particular superoxide, maintaining the reference properties of a Nafion-coated reference electrode. This cannot be mimicked easily in vitro due to superoxide's short lifetime in solution.

In conclusion, we have shown that we can prevent electrochemical shifts in fast cyclic voltammetric recordings that occur due to chronic implantation of reference electrodes with Nafion coatings on the Ag/AgCl surface. We linked the stability of our electrodes with the levels of Nafion on the electrode surface and showed that the Nafion stops cell adsorption onto the reference electrodes and maintains the integrity of the Ag/AgCl surface. We show several lines of evidence that strongly suggest that the shift is due to a change in the chemical microenvironment around the reference electrode because of the physical or chemical processes incurred by the glial scar.

METHODS

Animals and Surgery. Surgery for voltammetric measurements of dopamine were performed as previously described.[25] Briefly, male Sprague−Dawley rats aged 90−120 days (275−350 g) (Charles River Laboratories., Raleigh, NC) were anesthetized with urethane (1.5 g kg^{-1} rat weight) and affixed into a stereotaxic frame (Kofp instruments). Flat-skull surgical techniques were employed using coordinates from a stereotaxic atlas.[34] A guide cannula (Bioanalytical Systems, West Lafayette, IL) was implanted above the nucleus accumbens (NAc) (stereotaxic coordinates relative to bregma: 2.2 mm anterior, 1.7 mm lateral), and a bipolar stimulating electrode (Plastics One, Roanoke, VA) was lowered into the medial forebrain bundle (MFB) (stereotaxic coordinates relative to bregma: 1.8 mm posterior, 1.7 mm lateral, 8.5 mm ventral). Reference electrodes (prepared as described below) were implanted in the contralateral hemisphere. Electrodes were stabilized with skull screws and cranioplastic cement. After voltammetry and impedance experiments, acetone was applied to the cement around the reference electrode in order to soften it. A small crater was drilled around the reference electrode pin so that it could be removed carefully from the tissue, minimizing tissue damage. All surgical procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee and in concordance with the NIH Guide for the Care and Use of Animals.

Fast-Scan Cyclic Voltammetry. Carbon-fiber microelectrodes were used for voltammetric recordings of dopamine. T-650 carbon fibers (Thornel, Amoco Corp., Greenville, SC) were aspirated into glass capillaries (AM Systems, Sequim, WA), pulled on a vertical pipet puller (model PE-22, Narishige Group, Tokyo, Japan), and cut to a length of 50−100 μm. An electrochemical waveform was applied to the electrode that scanned from −0.4 to 1.3 V at a rate of 400 V s^{-1} at 10 Hz. The holding potential of −0.4 V between voltammetric scans allows for adsorption of dopamine to the electrode. Waveform application, current monitoring, and stimulus application were performed using a customized version of the TH-1 software (ESA, Chelmsford, MA) written in LabVIEW (National Instruments, Austin, TX) with a custom built potentiostat (UEI, University of North Carolina Chemistry Department Electronics Facility) for waveform application. A DAC/ADC card (NI 6251 M, National Instruments) and a triggering card (NI 6711, National Instruments) were used to interface between the software and the instrument and the timing of the electrical stimulation with the waveform application.

Flow Injection Analysis. Flow injection analysis was used for in vitro calibration experiments.[35] The carbon-fiber microelectrode was placed in the output of a six-port HPLC loop injector mounted on a two-position actuator (Rheodyne model 7010 valve and 5701 actuator), operated by a 12 V DC solenoid valve kit (Rheodyne, Rohnert Park, CA). The apparatus enabled the introduction of a rectangular pulse of analyte to the microelectrode surface using a syringe infusion pump (Harvard Apparatus model 940, Hollison, MA) at a flow rate of 2 mL/min. The flow injection buffer was constituted with the following: Trizma hydrochloride (15 mM), NaCl (140 mM), KCl (3.25 mM), $2H_2O \cdot CaCl$ (1.2 mM), $H_2O \cdot NaH_2PO_4$ (1.25 mM), $MgCl_2$ (1.2 mM), and Na_2SO_4 (2.0 mM), all purchased from Sigma-Aldrich (St. Louis, MO).

Nafion Coatings. Reference electrodes were fabricated using a modified procedure originally described by Moussy and Harrison.[24] Sintered Ag/AgCl reference electrodes (E255A, In Vivo Metric, Healdsburg, CA) were mounted and soldered into conductive pins. The entirety of the electrode was dipped in 5% Nafion solution (Liquion-1105-MeOH, Ion Power, DE) for 10 s using a slow agitating motion. This coating was allowed to dry for 30 min, and the process was repeated for a total of five coatings. The electrodes were air-dried overnight and then cured at 120 °C for 1 h. Because of the toxic nature of the methanol solvent, the curing process at 120 °C is essential to ensure full evaporation of the methanol from the thick Nafion membrane.

Immunohistochemistry. After completion of voltammetric and impedance experiments, rats were transcardially perfused with ice-cold 0.1 M PBS, pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were stored in 4% paraformaldehyde for 7 days and then sliced into 40 μM sections using a vibratome (VT1000S, Leica, Bannockburn, IL). Slices were washed three times for 10 min each in 0.1 M PBS and then incubated in Citra Antigen Retrieval Buffer (Biogenex, Freemont, CA) for 1 h at 70 °C. Slices were again washed in 0.1 M PBS and then incubated in 3% hydrogen peroxide for 10 min. Slices were then washed three additional times for 10 min each before being incubated in 10% goat serum with 0.075% Triton X-100 for 1 h at room temperature. Primary antibody (glial fibrillary acidic protein, GFAP; DAKO Chemical, Carpentieria, CA) was then applied to the slices at 1:1000 at 4 °C overnight. Primary antibody was removed from the slices by three washes of 0.1 M PBS. For light microscopy, secondary antibody (biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA) was then applied at 1:200 at room temperature for 1 h. Slices were washed three times in PBS, and avidin−biotin-horseradish peroxidase complex (ABC Elite kit; Vector Laboratories) was applied for 1 h at room temperature. Slices were then washed three more times in PBS and exposed to a

solution of 3,3′-diaminobenzidine (DAB; 0.5 mg/mL in PBS; Sigma-Aldrich, St. Louis, MO) for 1 min. DAB was removed by three additional washes with PBS. Slices were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), dried overnight, subjected to dehydration in graded ethanol followed by clearing in CitriSolv (Fisher Scientific), and finally coverslipped using Cytoseal (Richard Allen Scientific, Kalamazoo, MI). Micrographs were collected using an Olympus microscope attached to a Q-Imaging camera and controlled using BioQuant imaging software (Nashville, TN).

For fluorescence microscopy, hydrogen peroxide exposure was omitted and secondary antibody (AlexaFluor 600; Invitrogen, Carlsbad, CA) was applied at 1:200 at room temperature for 1 h in the dark. Secondary antibody was removed by three washes of PBS, and slices were wet-mounted on Superfrost Plus slides using Cytoseal. Fluorescence micrographs were obtained using the same setup as above.

SEM-EDS. Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDS) were performed on an S-4700 cold cathode field emission scanning electron microscope (Hitachi, Pleasanton, CA). SEM images were collected under high vacuum, using an excitation voltage of 20 kV. EDS data were collected using a Si (Li) detector and quantified using the INCA PentaFET -x3 software (Oxford Instruments, Concord, MA), and calibrated using 99% Cu. EDS spectra were collected at three distinct 300 × 300 μm^2 locations on each electrode, and the values for atom % fluorine and chlorine were averaged.

Impedance Measurements. Impedance measurements were performed in anesthetized animals immediately after voltammetric measurements using a Hewlett-Packard 4284A precision LCR meter with Agilent 16048A test leads (Santa Clara, CA). The instrument was PC operated via a USB-GPIB interface with in-house LabView based (National Instruments, Austin, TX) software (UNC Chemistry Electronics Facility). Impedance for Nafion-coated and bare Ag/AgCl reference electrodes was measured versus an acutely implanted 1 cm long Ag/AgCl electrode. Impedance spectra for perturbation of 50 mV for frequencies from 20 Hz to 200 kHz were recorded.

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Author Contributions

Parastoo Hashemi, Paul Walsh, Thomas Guillot, Julie Gras-Najar and Pavel Takmakov conducted the experiments. Parastoo Hashemi, Paul Walsh, Fulton Crews, and R. Mark Wightman planned the experiments. Parastoo Hashemi, Paul Walsh, and R. Mark Wightman wrote the manuscript.

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ABBREVIATIONS

Ag/AgCl, silver/silver chloride reference electrode; SEM, scanning electron microscopy; EDS, energy dispersive X-ray spectroscopy; GFAP, glial fibrillary acid protein; FSCV, fast-scan cyclic voltammetry

REFERENCES

(1) Kuhr, W. G., Ewing, A. G., Caudill, W. L., and Wightman, R. M. (1984) Monitoring the stimulated release of dopamine with in vivo voltammetry. I: Characterization of the response observed in the caudate nucleus of the rat. *J. Neurochem. 43*, 560–569.

(2) Ewing, A. G., and Wightman, R. M. (1984) Monitoring the stimulated release of dopamine with in vivo voltammetry. II: Clearance of released dopamine from extracellular fluid. *J. Neurochem. 43*, 570–577.

(3) Hashemi, P., Dankoski, E. C., Petrovic, J., Keithley, R. B., and Wightman, R. M. (2009) Voltammetric detection of 5-hydroxytryptamine release in the rat brain. *Anal. Chem. 81*, 9462–9471.

(4) Park, J., Kile, B. M., and Wightman, R. M. (2009) In vivo voltammetric monitoring of norepinephrine release in the rat ventral bed nucleus of the stria terminalis and anteroventral thalamic nucleus. *Eur. J. Neurosci. 30*, 2121–2133.

(5) Cheer, J. F., Aragona, B. J., Heien, M. L., Seipel, A. T., Carelli, R. M., and Wightman, R. M. (2007) Coordinated accumbal dopamine release and neural activity drive goal-directed behavior. *Neuron 54*, 237–244.

(6) Owesson-White, C. A., Cheer, J. F., Beyene, M., Carelli, R. M., and Wightman, R. M. (2008) Dynamic changes in accumbens dopamine correlate with learning during intracranial self-stimulation. *Proc. Natl. Acad. Sci. U.S.A. 105*, 11957–11962.

(7) Takmakov, P., Zachek, M. K., Keithley, R. B., Walsh, P. L., Donley, C., McCarty, G. S., and Wightman, R. M. (2010) Carbon microelectrodes with a renewable surface. *Anal. Chem. 82*, 2020–2028.

(8) Garris, P. A., and Wightman, R. M. (1994) Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. *J. Neurosci. 14*, 442–450.

(9) Robinson, D. L., Venton, B. J., Heien, M. L., and Wightman, R. M. (2003) Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. *Clin. Chem. 49*, 1763–1773.

(10) Nicolelis, M. A., Dimitrov, D., Carmena, J. M., Crist, R., Lehew, G., Kralik, J. D., and Wise, S. P. (2003) Chronic, multisite, multielectrode recordings in macaque monkeys. *Proc. Natl. Acad. Sci. U.S.A. 100*, 11041–11046.

(11) Rousche, P. J., and Normann, R. A. (1998) Chronic recording capability of the Utah Intracortical Electrode Array in cat sensory cortex. *J. Neurosci. Methods 82*, 1–15.

(12) Williams, J. C., Rennaker, R. L., and Kipke, D. R. (1999) Long-term neural recording characteristics of wire microelectrode arrays implanted in cerebral cortex. *Brain Res. Protoc. 4*, 303–313.

(13) Stensaas, S. S., and Stensaas, L. J. (1976) The reaction of the cerebral cortex to chronically implanted plastic needles. *Acta Neuropathol. 35*, 187–203.

(14) Smith, G. M., Miller, R. H., and Silver, J. (1986) Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. *J. Comp. Neurol. 251*, 23–43.

(15) Szarowski, D. H., Andersen, M. D., Retterer, S., Spence, A. J., Isaacson, M., Craighead, H. G., Turner, J. N., and Shain, W. (2003) Brain responses to micro-machined silicon devices. *Brain Res. 983*, 23–35.

(16) Polikov, V. S., Tresco, P. A., and Reichert, W. M. (2005) Response of brain tissue to chronically implanted neural electrodes. *J. Neurosci. Methods 148*, 1–18.

(17) Kreutzberg, G. W. (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci. 19*, 312–318.

(18) Biran, R., Martin, D. C., and Tresco, P. A. (2005) Neuronal cell loss accompanies the brain tissue response to chronically implanted silicon microelectrode arrays. *Exp. Neurol. 195*, 115–126.

(19) Turner, J. N., Shain, W., Szarowski, D. H., Andersen, M., Martins, S., Isaacson, M., and Craighead, H. (1999) Cerebral

astrocyte response to micromachined silicon implants. *Exp. Neurol. 156*, 33–49.

(20) Edell, D. J., Toi, V. V., McNeil, V. M., and Clark, L. D. (1992) Factors influencing the biocompatibility of insertable silicon microshafts in cerebral cortex. *IEEE Trans. Biomed. Eng. 39*, 635–643.

(21) Rousche, P. J., Pellinen, D. S., Pivin, D. P., Jr., Williams, J. C., Vetter, R. J., and Kipke, D. R. (2001) Flexible polyimide-based intracortical electrode arrays with bioactive capability. *IEEE Trans. Biomed. Eng. 48*, 361–371.

(22) George, P. M., Lyckman, A. W., LaVan, D. A., Hegde, A., Leung, Y., Avasare, R., Testa, C., Alexander, P. M., Langer, R., and Sur, M. (2005) Fabrication and biocompatibility of polypyrrole implants suitable for neural prosthetics. *Biomaterials 26*, 3511–3519.

(23) Yuen, T. G., and Agnew, W. F. (1995) Histological evaluation of polyesterimide-insulated gold wires in brain. *Biomaterials 16*, 951–956.

(24) Moussy, F., and Harrison, D. J. (1994) Prevention of the Rapid Degradation of Subcutaneously Implanted Ag/Agcl Reference Electrodes Using Polymer-Coatings. *Anal. Chem. 66*, 674–679.

(25) Phillips, P. E., Robinson, D. L., Stuber, G. D., Carelli, R. M., and Wightman, R. M. (2003) Real-time measurements of phasic changes in extracellular dopamine concentration in freely moving rats by fast-scan cyclic voltammetry. *Methods Mol. Med. 79*, 443–464.

(26) Kristensen, E. W., Kuhr, W. G., and Wightman, R. M. (1987) Temporal characterization of perfluorinated ion exchange coated microvoltammetric electrodes for in vivo use. *Anal. Chem. 59*, 1752–1757.

(27) Wiedemann, D. J., Basse-Tomusk, A., Wilson, R. L., Rebec, G. V., and Wightman, R. M. (1990) Interference by DOPAC and ascorbate during attempts to measure drug-induced changes in neostriatal dopamine with Nafion-coated, carbon-fiber electrodes. *J. Neurosci. Methods 35*, 9–18.

(28) Jaquins-Gerstl, A., and Michael, A. C. (2009) Comparison of the brain penetration injury associated with microdialysis and voltammetry. *J. Neurosci. Methods 183*, 127–135.

(29) Keithley, R. B., Takmakov, P., Bucher, E. S., Belle, A. M., Owesson-White, C. A., Park, J., and Wightman, R. M. (2011) Higher sensitivity dopamine measurements with faster-scan cyclic voltammetry. *Anal. Chem. 83*, 3563–3571.

(30) Frampton, J. P., Hynd, M. R., Shuler, M. L., and Shain, W. (2010) Effects of glial cells on electrode impedance recorded from neuralprosthetic devices in vitro. *Ann. Biomed. Eng. 38*, 1031–1047.

(31) Roitbak, T., and Sykova, E. (1999) Diffusion barriers evoked in the rat cortex by reactive astrogliosis. *Glia 28*, 40–48.

(32) Zielasek, J., Muller, B., and Hartung, H. P. (1996) Inhibition of cytokine-inducible nitric oxide synthase in rat microglia and murine macrophages by methyl-2,5-dihydroxycinnamate. *Neurochem. Int. 29*, 83–87.

(33) Giulian, D., and Baker, T. J. (1986) Characterization of ameboid microglia isolated from developing mammalian brain. *J. Neurosci. 6*, 2163–2178.

(34) Paxinos, G., and Watson, C. (2007) *The Rat Brain in Stereotaxic Coordinates*, 6th ed., Elsevier: Amsterdam.

(35) Kristensen, E. W., Wilson, R. L., and Wightman, R. M. (1986) Dispersion in Flow-Injection Analysis Measured with Microvoltammetric Electrodes. *Anal. Chem. 58*, 986–988.